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14. ABSTRACT
An immunopathology reported in some autistic patients is development of autoantibodies against brain-specific proteins, suggesting impacts to regulatory T cells (Tregs). Perfluorooctanoic acid (PFOA) and perfluorooctane sultanate (PFOS) are environmental pollutants that induce developmental and immunotoxicity. Our hypothesis is that developmental exposure to PFOA or PFOS will affect number and/or function of Tregs and increase autoimmune risk in offspring. In immunocompetent male and female offspring exposed to PFOA or PFOS during gestation and lactation, splenic Treg number and function, serum markers of autoreactivity, and levels of myelin basic protein and T cell infiltration in the cerebella were evaluated. Notable findings included alterations to splenic Treg number and splenic Treg ex vivo function after developmental exposure to either compound. These data suggest that the number of Tregs or the functional capacity of Tregs may be altered by developmental exposure to PFOA of PFOS. However, autism risk cannot be determined from these data alone.

15. SUBJECT TERMS
Regulatory T cells, immunophenotyping, autoantibodies, CD3+, myelin basic protein, autism

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INTRODUCTION

The etiology of autism and related neurodevelopmental disorders is largely unknown. Myriad hypotheses have suggested that exogenous agents, such as environmental pollutants, play a role in causing or triggering dysfunctional development that may culminate in an autism diagnosis. It also has been suggested that immunopathogenesis, or alterations to the immune system, occur in subsets of autistic patients. One type of immunopathology that has been reported in some autistic patients is the development of autoantibodies against brain-specific proteins, which suggests that regulatory T cells (Tregs) or central/peripheral tolerance (the process by which autoreactive T cells are isolated or eliminated) may be impacted. The emerging contaminants perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) are environmentally pervasive and have been associated with both developmental toxicity and immunotoxicity. It is therefore plausible that exposure to these compounds may affect Tregs or central/peripheral tolerance and may result in dysregulation of autoreactive T cells and subsequent neural damage. Our hypothesis is that developmental exposure to PFOA or PFOS will affect the number and/or function of regulatory T cells (Tregs) and lead to changes indicative of increased autoimmune risk in offspring. In immunocompetent male and female offspring that were exposed to PFOA or PFOS during gestation and lactation, we evaluated splenic Treg number and function, serum levels of anti-MBP, anti-dsDNA, and anti-ssDNA, and levels of myelin basic protein and T cell infiltration in the cerebella. The findings of our studies will establish whether PFOA and/or PFOS impact Tregs and/or tolerance when given during of pre- and post-natal development and create a system that may lead to autoimmune reactions against neural tissues.

BODY

Specific Aim #1: To determine the effects of developmental exposure to perfluorooctanoic acid (PFOA) or perfluorooctane sulfonate (PFOS) on regulatory T cells (Tregs; CD4+CD25+FoxP3+) and autoantibody production in C57Bl/6 mice by assessing Treg number with flow cytometry, Treg function by evaluation of IL-10 and perforin* secretion ex vivo, and markers of autoreactivity with serum anti-MBP (myelin basic protein), anti-ssDNA, and anti-dsDNA.

> *Note: Note perforin was measured by flow cytometry from cells cultured ex vivo. However, perforin levels in cultured Treg cells were too low to be detected. Both perforin and granzyme B are secreted by Tregs to induce cell death of host immune cells. However, although we cultured Tregs with IL-2, we believe that they were not sufficiently activated to produce a level of perforin in culture that we could detect with flow cytometry. Alternatively, low levels of perforin in cultured Tregs could indicate insufficient function of Tregs induced by developmental PFOA or PFOS exposure. Additional research is required to verify such functional defects.

Tasks 1 and 2. Developmental effects of PFOA exposure in C57Bl/6 mice. All studies completed. Data analysis completed. Manuscript to be submitted to *NeuroToxicology* on February 15, 2012.

Tasks 3 and 4. Developmental effects of PFOS exposure in C57Bl/6 mice. All studies completed. Data analysis completed. Manuscript to be submitted to *Journal of Toxicology and Environmental Health* by April of 2012.

Results of Tasks 1 through 4

In four experiments, 96 dams were gavaged with PFOA (DoD-1 and DoD-2) and 96 dams were gavaged with PFOS (DoD-4 and DoD-4) from pairing with males through weaning of pups, which exposed offspring from gestational day zero (GD0) through weaning, at postnatal day 21 (PND21). The following endpoints were collected in subsets of the adult offspring:

- Number of splenic Tregs (CD4+CD25+FoxP3+) as determined by flow cytometry
- Ex vivo Treg (CD4+CD25+FoxP3+) function as measured by IL-10 release
- Serum markers of autoreactivity (anti-dsDNA, anti-ssDNA, and anti-MBP)

Reproductive and developmental outcomes

Pregnancy success of dams exposed to PFOA was 67%, 46%, 54%, and 58% for the 0 mg/kg, 0.02 mg/kg, 0.2 mg/kg, and 2 mg/kg dose groups, respectively (Table 1A). Pregnancy success of dams exposed to PFOS was 58%, 54%, 50%, and 58% for the 0 mg/kg, 0.02 mg/kg, 0.2 mg/kg, and 2 mg/kg dose groups, respectively (Table 2B). For each chemical, pregnancy success rates did not differ by dose ($P < 0.05$) and were appropriate for the C57Bl/6 strain of mouse. The number of litters delivered and number of litters weaned (Tables 1A and 1B) did not differ statistically ($P < 0.05$) by dose, which indicates that the administered PFOA and PFOS doses were not overtly fetally toxic.

Table 1A. Reproductive outcome of dams dosed with PFOA via gavage from pairing with males through weaning of offpsring. DoD-1 and DoD-2 studies combined. N = 24 dams/dose group.

	0 mg/kg PFOA	**0.02 mg/kg PFOA**	**0.2 mg/kg PFOA**	**2 mg/kg PFOA**
Dams pregnant	16	11	13	14
Litters delivered	13	11	13	14
Litters weaned	7	9	11	9

Table 1B. Reproductive outcome of dams dosed with PFOS via gavage from pairing with males through weaning of offspring. DoD-3 and DoD-4 studies combined. N = 24 dams/dose group.

	0 mg/kg PFOA	**0.02 mg/kg PFOA**	**0.2 mg/kg PFOA**	**2 mg/kg PFOA**
Dams pregnant	14	13	12	14
Litters delivered	14	11	11	14
Litters weaned	13	11	9	12

Developmental exposure to PFOA did affect the weight of litters (Figure 1A). Litters from the dams exposed to 2 mg/kg of PFOA had statistically lower body weights ($P < 0.05$) throughout the lactational period, from birth at PND1 through weaning at PND21. Litter weights in the 2 mg/kg dose were reduced relative to litter weights in the control group by 17.3%, 49.8%, 30.6%, and 32.7% at PND1, PND9, PND16, and PND21, respectively. Although we did not monitor developmental milestones in the study, anecdotally, we noticed that pups from these litters were visibly runted and developed hair and opened eyes at a later age compared to other dose groups and the control group. Five of the nine litters from this group were weaned at 23-27 days of age rather than 21 days of age due to delays in development. Developmental exposure to PFOS had no statistical effects on litter weights (Figure 1A).

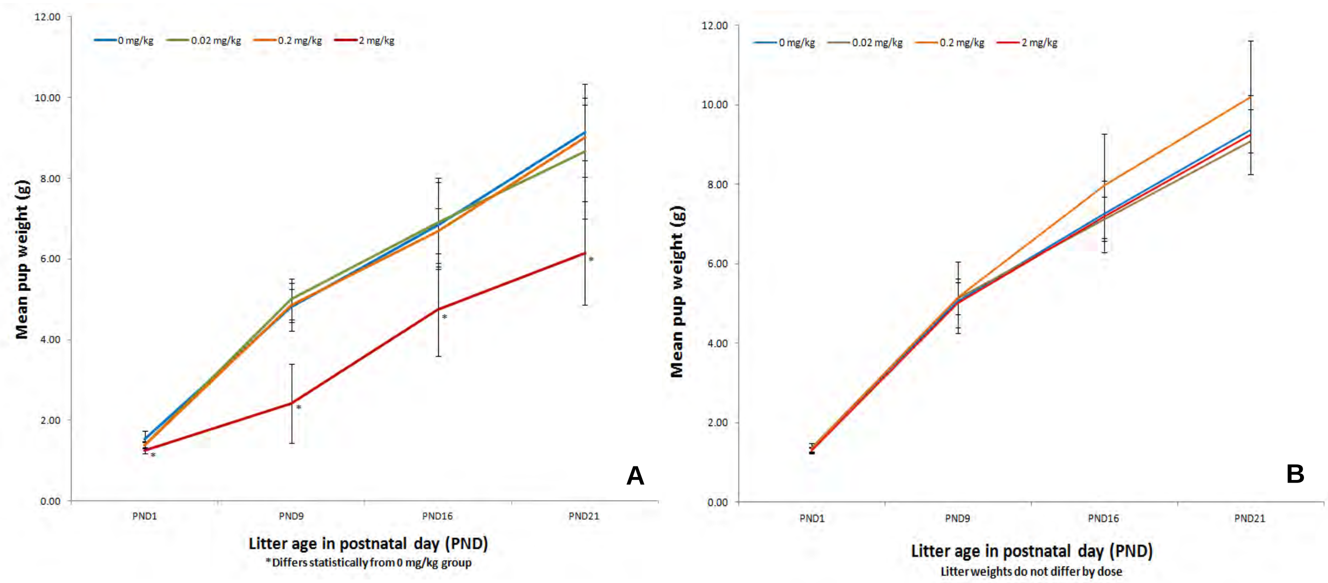

Figure 1. Mean (± standard deviation) litter weights in offspring of dams dosed with PFOA (A) or PFOS (B) via gavage from pairing with males through weaning of offspring. Offspring of dams exposed to 2 mg/kg of PFOA weighed 32.6% less, on average, from offspring of control dams from birth (PND1) through weaning (PND21). Offspring of dams exposed to PFOS did not differ statistically by litter weight.

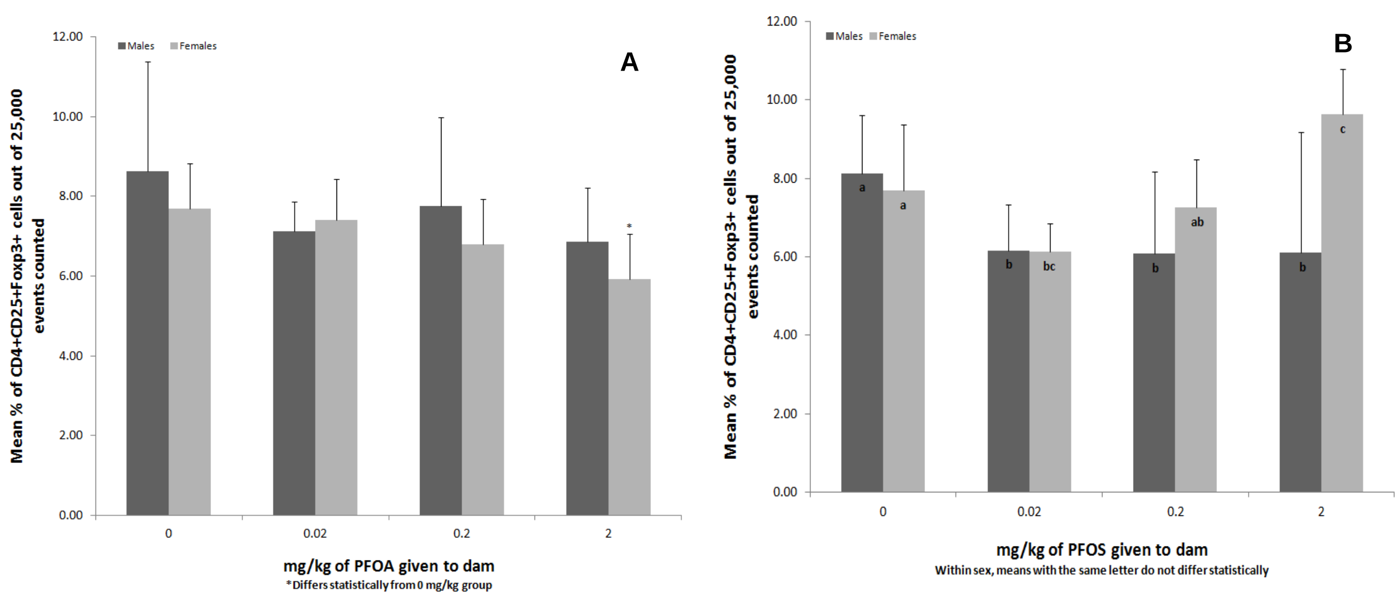

Figure 2. Mean percent (± standard deviation) of CD4+CD25+Foxp3+ regulatory T cells (Tregs) in offspring of dams dosed with PFOA (A) or PFOS (B) via gavage from pairing with males through weaning of offspring. PFOA suppressed Treg numbers in female offspring exposed to 2 mg/kg; PFOS suppressed Treg numbers in male offspring at all doses.

Immunophenotyping
The number of splenic CD4+CD25+Foxp3+ Tregs were counted in subsets of offspring (Figures 2A and 2B). Single cell suspensions were made from spleens of individual animals, stained for CD4+, CD25+, and Foxp3+, and counted in an Accuri flow cytometer. The resulting percentage of cells positive for CD4, CD25, and Foxp3 were determined from 25,000 events (cells). The percent of T cells (Tregs) with this phenotype in spleens from the control group was approximately 8%, which was expected for this subset of T cells. Spleens of offspring from dams treated with PFOA (Figure 2A) had slightly lower Treg numbers relative to spleens of offspring from control dams; however, only spleens of females from the 2 mg/kg dose had statistically lower numbers of Tregs (5.9%; $P < 0.05$). In offspring of dams treated with PFOS, spleens of males from all dose groups had 25% fewer Tregs relative to spleens of offspring from control dams ($P< 0.05$). In spleens of offspring from dams treated with PFOS, females from the 0.02 mg/kg group had 20% fewer Tregs relative to females from the control group and females from the 2 mg/kg group had 25% more Tregs relative to females from the control group.

Ex vivo Treg function
The ability of splenic CD4+CD25+ Tregs to release IL-10 in culture with target cells was determined from subsets of offspring. Single cell suspensions were made from pooled spleens of five representative males and five representative females from each dose group. If possible, offspring from the same dams were not pooled unless it was not possible due to low litter numbers for a particular dose. Briefly, CD4+CD25+ Tregs were isolated from pooled spleen cells and co-cultured ex vivo for 72 hours with CD4+CD25- T cells as targets, and stimulated with anti-CD3+, anti-CD28+, and IL-2 in RPMI culture medium. IL-10 levels were measured in the culture medium with an R&D Systems ELISA kit. Results are shown in Figure 3. In offspring from dams exposed to PFOA (A), IL-10 released from Tregs cultured from male offspring was decreased by 61%, 75%, and 75%, in the 0.02 mg/kg, 0.2 mg/kg, and 2 mg/kg dose groups, respectively ($P < 0.05$). In female offspring, levels of IL-10 released from cultured Tregs were increased by 204% in the 0.02 mg/kg dose group relative to controls ($P < 0.05$); no other differences were detectable in the female offspring. In offspring from dams exposed to PFOS (B) IL-10 released from Tregs cultured from male offspring was increased by 240% in the 0.02 mg/kg dose group and decreased by 72% in the 0.2 mg/kg dose group ($P < 0.05$). Relative to the control group, IL-10 released from Tregs cultured from female offspring was decreased by 37.6% in the 0.02 mg/kg dose group and increased by 192.6% in the 0.2 mg/kg group ($P < 0.05$).

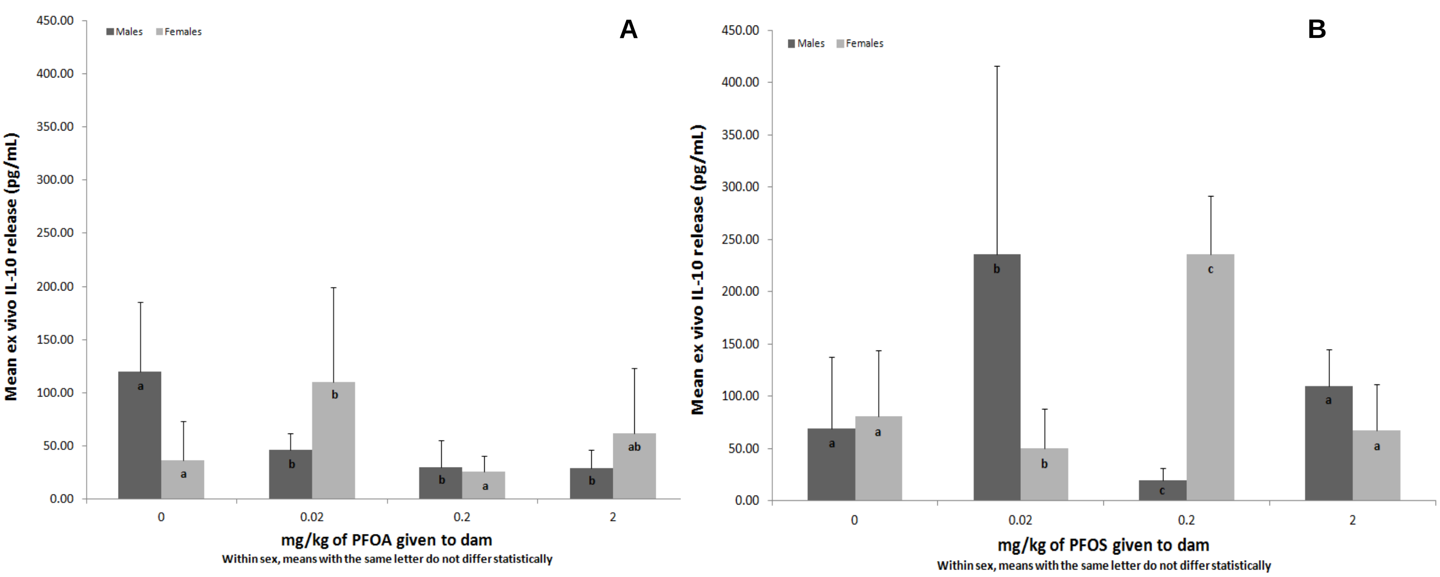

Figure 3. Mean IL-10 (± standard deviation) released (pg/mL) from regulatory T cells (Tregs; CD4+CD25+Foxp3+) isolated from offspring of dams dosed with PFOA (A) or PFOS (B) via gavage from pairing with males through weaning of offspring. Tregs were co-cultured ex vivo for 72 hours with CD4+CD25- T cells and stimulated with anti-CD3+, anti-CD28+, and IL-2 in RPMI culture medium. PFOA suppressed IL-10 from Tregs cultured from male offspring at all doses and elevated IL-10 from Tregs cultured from female offspring exposed to 0.02 mg/kg. PFOS elevated IL-10 from Tregs cultured from male offspring exposed to 0.02 mg/kg and suppressed IL-10 from Tregs cultured from male offspring exposed to 0.2 mg/kg. The opposite was observed from IL-10 released from Tregs cultured from female offspring.

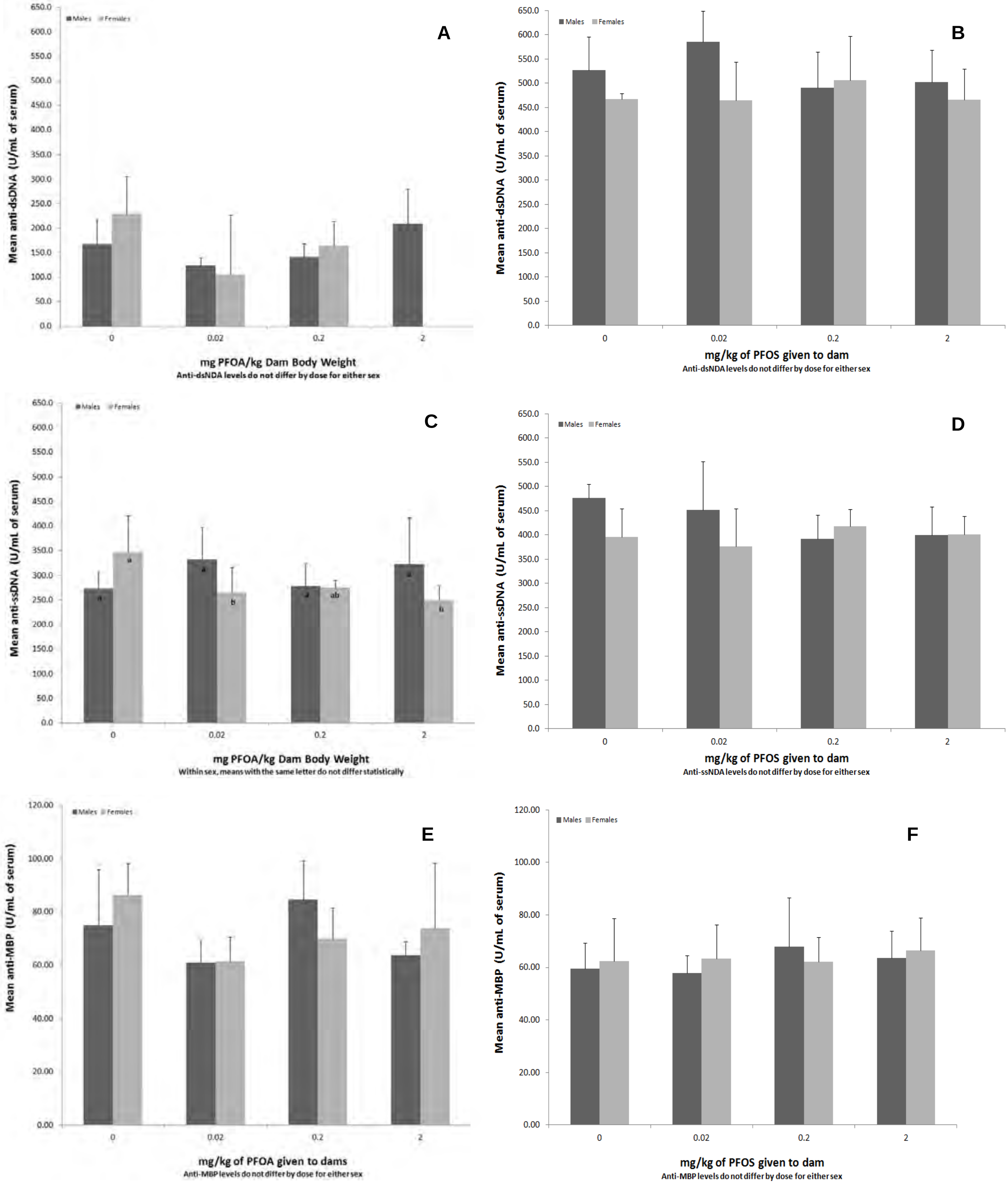

Figure 4. Mean serum (± standard deviation) markers of autoreactivity in offspring of dams dosed with PFOA or PFOS via gavage from pairing with males through weaning of offspring. With the exception of a slight decrease in anti-ssDNA in female offspring exposed to 0.02 and 2 mg/kg of PFOA (C), levels of serum markers for autoreactivity did not differ statistically by dose for either chemical. A) anti-dsDNA of offspring exposed to PFOA; B) anti-dsDNA of offspring exposed to PFOS; C) anti-ssDNA of offspring exposed to PFOA; D) anti-ssDNA of offspring exposed to PFOS; E) anti-myelin basic protein of offspring exposed to PFOA; and F) anti-myelin basic protein of offspring exposed to PFOS Note: no samples were available for female offspring exposed to 2 mg/kg of PFOA for measurement of anti-dsDNA (A).

Serum markers of autoreactivity
Serum markers of autoreactivity were measured in subsets of offspring. Serum markers included levels of anti-dsDNA, anti-ssDNA, and anti-myelin basic protein (anti-MBP). Figure 4 illustrates the results of the three different assays. With the exception of a 26% decrease, on average, in anti-ssDNA in female offspring exposed to 0.02 and 2 mg/kg of PFOA (C), no differences in autoreactivity markers were detected among dose groups or between sexes for either chemical.

Specific Aim #2: To determine the effects of developmental exposure to perfluorooctanoic acid (PFOA) or perfluorooctane sulfonate (PFOS) on infiltration of T cells into the brains of C57Bl/6 mice by staining brain cerebellar sections with anti-CD3 and anti-MBP (myelin basic protein).

Tasks 1 and 2. Developmental effects of PFOA exposure in C57Bl/6 mice. All studies completed. Data analysis completed. Manuscript to be submitted to *NeuroToxicology* on February 15, 2012.

Tasks 3 and 4. Developmental effects of PFOS exposure in C57Bl/6 mice. All studies completed. Data analysis ongoing. Manuscript to be submitted to *Journal of Toxicology and Environmental Health* by April of 2012.

Results of Tasks 1 through 4
In the four experiments that were performed under Specific Aim 1, the following endpoints were collected in subsets of the adult offspring:

- Measurement of T cell infiltration into cerebella of exposed offspring
- Measurement of levels of myelin basic protein (MBP) in cerebella of exposed offspring

T cell infiltration into cerebella of exposed offspring
Numbers of CD3+ T cells that had infiltrated brains were counted in cerebella of subsets of offspring. Briefly, cerebella were immersion fixed in 10% neutral buffered formalin for 24 hours, paraffin-embedded, and sliced at 6 µm with a rotary microtome. Sections were stained immunohistochemically with anti-CD3+ antibody and the number of cells stained with CD3+ were counted. A representative section is displayed in Figure 5. No CD3+ T cell infiltration was observed in any of the examined sections.

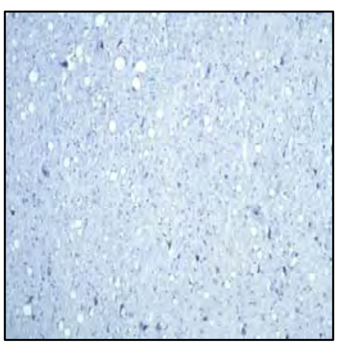

Figure 5. A representative cerebellar section from a female offspring of a dam dosed with PFOA via gavage from pairing with males through weaning of pups. No CD3+ T cell infiltration was observed in any of the examined sections (magnification = 20X) from PFOA- or PFOS-exposed offspring

Levels of myelin basic protein (MBP) in cerebella of exposed offspring
Relative MBP levels were measured in cerebella or subsets of offspring. Cerebella were prepared as described above, but were stained immunohistochemically with anti-MBP antibody rather than anti-CD3+. The relative levels of MBP were determined and scored as normal, mildly depleted, moderately depleted, or severely depleted. The average level of MBP depletion did not differ by dose for either chemical (Figures 6 and 7).

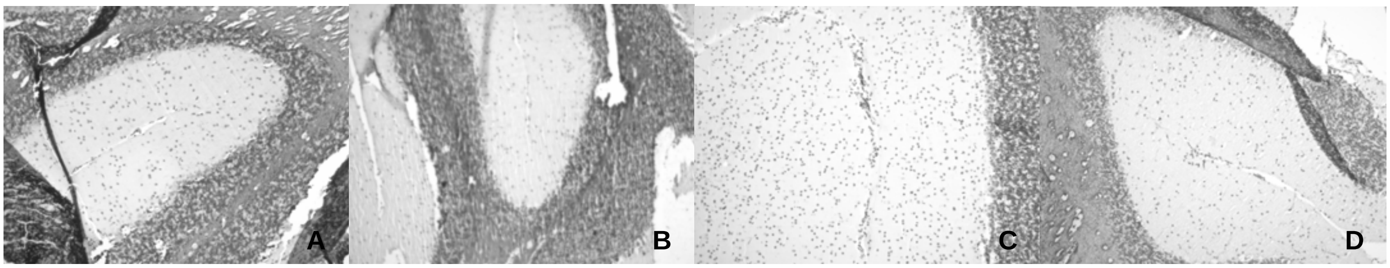

Figure 6. Sample sections of cerebella from offspring exposed to PFOA used to determine relative intensity of myelin basic protein (MBP) staining. (A) 0 mg/kg; (B) 0.02 mg/kg; (C) 0.2 mg/kg; (D) 2 mg/kg. Magnification = 20X. No statistical difference in the intensity of MBP staining was detected among dose groups.

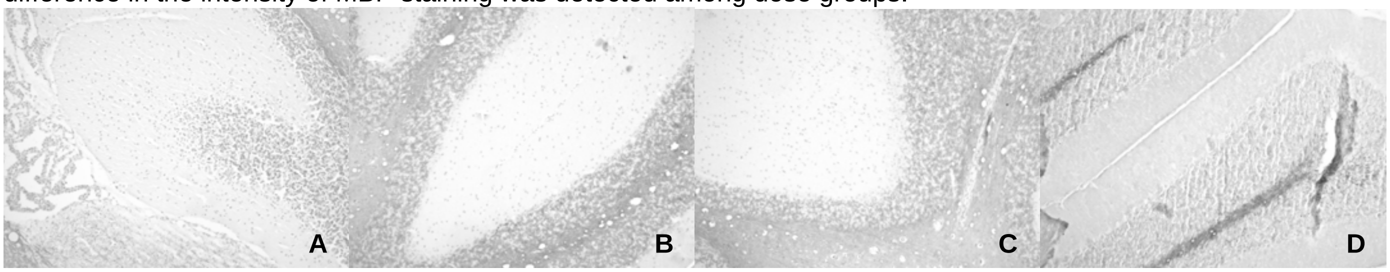

Figure 7. Sample sections of cerebella from offspring exposed to PFOS used to determine relative intensity of myelin basic protein (MBP) staining. (A) 0 mg/kg; (B) 0.02 mg/kg; (C) 0.2 mg/kg; (D) 2 mg/kg. Magnification = 20X. No statistical difference in the intensity of MBP staining was detected among dose groups.

Summary of findings for Tasks 1 through 4 of Specific Aim #1

<u>PFOA</u>: Litter weights of offspring from dams exposed to 2 mg/kg of PFOA were reduced by 32.6%, on average, from PND1 through weaning (PND21). Splenic Treg numbers of female offspring from dams exposed to 2 mg/kg of PFOA were reduced by 5.9%. Male C57Bl/6 mice given PFOA from gestation through lactation via exposure to dams exhibited a statistically significant decrease in the amount of IL-10 released from splenic Tregs cultured ex vivo. Levels IL-10 released from splenic Tregs cultured ex vivo was elevated in female offspring from dams exposed to 0.02 mg/kg PFOA. Anti-ssDNA, a serum marker of autoreactivity, was decreased by 26%, on average, in female offspring from dams exposed to 0.02 and 2 mg/kg PFOA. No other endpoints were statistically different by dose for offspring exposed to PFOA.

<u>PFOS</u>: Splenic Treg numbers from male offspring of dams exposed to all doses of PFOS were reduced by 25%, on average. Splenic Treg numbers from female offspring of dams exposed to 0.02 or 2 mg/kg of PFOA varied statistically from the control group values. Levels IL-10 released from splenic Tregs cultured ex vivo was elevated in male offspring of dams exposed to 0.02 mg/kg PFOS and in female offspring of dams exposed to 0.2 mg/kg PFOS. Oppositely, levels of IL-10 released from splenic Tregs cultured ex vivo was reduced in male offspring of dams exposed to 0.2 mg/kg PFOS and in female offspring of dams exposed to 0.02 mg/kg PFOS. No other endpoints were statistically different by dose for offspring of dams exposed to PFOS.

Summary of findings for Tasks 1 and 2 of Specific Aim #2

Brains of C57Bl/6 mice given PFOA of PFOS from gestation through lactation via exposure to dams did not have T cell infiltration or depletion of levels of MBP.

KEY RESEARCH ACCOMPLISHMENTS

- Four developmental studies completed, two with perfluorooctanoic acid (PFOA) and two with perflurooctane sulfonate (PFOS), and each involving exposure to 48 dams (192 dams total).
- Endpoints were assessed in 431 offspring.
- Numbers of CD4+CD25+Foxp3+ regulatory T cells (Tregs) in spleens of exposed offspring were successfully counted with flow cytometery.
- Ex vivo function of CD4+CD25+ Tregs cultured from spleens of exposed offspring was successfully evaluated with IL-10 release.
- Serum markers of autoreactivity, including anti-dsDNA, anti-ssDNA, and anti-myelin basic protein (MBP) were successfully measured.
- Numbers of CD3+ T cells that had infiltrated brains of exposed offspring were successfully counted in cerebellar sections.
- The level of MBP depletion was successfully evaluated in cerebellar sections of exposed offspring.

REPORTABLE OUTCOMES

- One manuscript is in preparation for submission to *NeuroToxicology* (perfluorooctanoic acid [PFOA] results) and one manuscript is in submission to *Journal of Toxicology and Environmental Health* (perfluorooctane sulfonate [PFOS] results).

- Two high school students through the Pitt County Schools Honors Medicine Program (unpaid, volunteer positions) presented findings on T cell infiltration and myelin basic protein levels in brains of offspring exposed to perfluorooctanoic acid (PFOA) at their end-of-program conference in May of 2011.

- One high school student through the Summer Ventures Program (unpaid, volunteer position) presented findings on T cell infiltration in brains of offspring exposed to perfluorooctane sulfonate (PFOS) in July of 2011.

- One undergraduate student (paid through non-grant funds) presented findings on T cell infiltration and myelin basic protein levels in brains of offspring exposed to perfluorooctane sulfonate (PFOS) at the annual NeuroToxicology Conference in October/November of 2011. These findings will also be presented at East Carolina University's annual research week in April of 2012.

- The principal investigator presented findings related to perfluorooctanoic acid (PFOA) at the annual NeuroToxicology Conference in October/November of 2011.

- The principal investigator will present findings related perfluorooctane sulfonate (PFOS) at the annual Society of Toxicology conference in March of 2012.

- A National Institutes of Health R21 grant will be submitted in October of 2012. The application will be based on findings from the research accomplished through this grant project and will focus on mechanisms of immune suppression induced by developmental exposure to these compounds.

CONCLUSIONS

Specific Aim #1: To determine the effects of developmental exposure to perfluorooctanoic acid (PFOA) or perfluorooctane sulfonate (PFOS) on regulatory T cells (Tregs; CD4+CD25+FoxP3+) and autoantibody production in C57Bl/6 mice by assessing Treg number with flow cytometry, Treg function by evaluation of IL-10 and perforin secretion ex vivo, and markers of autoreactivity with serum anti-MBP (myelin basic protein), anti-ssDNA, and anti-dsDNA.

Specific Aim #2: To determine the effects of developmental exposure to perfluorooctanoic acid (PFOA) or perfluorooctane sulfonate (PFOS) on infiltration of T cells into the brains of C57Bl/6 mice by staining brain cerebellar sections with anti-CD3 and anti-MBP (myelin basic protein).

Conclusions of experiments for Specific Aim #1

Little is known about how regulatory T cells (Tregs) impact normal neural development or how disruptions to Tregs by environmental contaminants may alter neural development. Tregs may be part of a regulatory process that tips the balance toward disease or health. In this project, female C57Bl/6 mice were given perfluorooctanoic acid (PFOA) or perfluorooctane sulfonate (PFOS) from pairing with males through weaning of offspring to ensure that offspring were exposed throughout gestation and lactation. Endpoints assessed included: direct counts of splenic Treg numbers; direct evaluation of the ability of splenic Tregs to release suppressive cytokines by measuring ex vivo concentrations of IL-10; indirect evaluation of the ability of splenic Tregs to control autoreactive antibodies by measuring serum autoantibodies, T cell infiltration into brains, and levels of MBP in brains.

Effects of gestational and lactational exposure to 0.02 mg/kg, 0.2 mg/kg, or 2 mg/kg of PFOA or PFOS resulted in the following statistically significant ($P < 0.05$) changes:

- A reduction in litter weights by 32.6%, on average, in offspring from dams exposed to 2 mg/kg of PFOA. This reduction was observed from PND1 through weaning at PND21.
- A reduction (5.9%) in Treg numbers from female offspring of dams exposed to 2 mg/kg of PFOA, a reduction (25% on average) in Treg numbers from male offspring of dams exposed to all doses of PFOS, a reduction (20%) in Treg numbers from female offspring of dams exposed to 0.02 mg/kg of PFOS, and a 25% increase in Treg numbers from female offspring of dams exposed to 2 mg/kg of PFOS.
- A 70% reduction, on average, of ex vivo IL-10 release from splenic Tregs cultured from male offspring of dams exposed to all doses of PFOA, a 204% elevation of ex vivo IL-10 release from splenic Tregs cultured from female offspring of dams exposed to 0.02 mg/kg PFOA, a 240% increase and a 72% decrease in ex vivo IL-10 release from splenic Tregs cultured from male offspring of dams exposed to 0.02 and 0.2 mg/kg PFOS, respectively, and a 37.6% decrease and a 192.6% increase in ex vivo IL-10 release from splenic Tregs cultured from female offspring of dams exposed to 0.02 and 0.2 mg/kg PFOS, respectively.
- A 26% decrease in anti-ssDNA, a serum marker of autoreactivity, in female off spring of dams exposed to 0.02 and 2 mg/kg of PFOA.

Conclusions of experiments for Specific Aim #2

Brains of C57Bl/6 mice given PFOA of PFOS from gestation through lactation via exposure to dams did not have T cell infiltration or depletion of levels of MBP.

Implications of these findings

The most notable finding of our research is that developmental exposure to either PFOA of PFOS alters the ability of splenic Tregs cultured ex vivo to release IL-10 and alters the numbers of Tregs found in the spleen. Additional studies are required to further delineate the relationship between Treg number and ex vivo IL-10 release as the changes do not mirror one another. For example, male offspring of dams exposed to all doses of PFOA had reduced IL-10 release, but no changes in Treg numbers, which suggests a dysfunction in the ability of Tregs to release IL-10. While male offspring of dams exposed to 0.02 mg/kg of PFOS had a reduction in Treg numbers, but an increase in IL-10 release, male offspring of dams exposed to 0.2 mg/kg of PFOS had both a reduction in Treg number and a reduction in IL-10 release, which suggests that the reduction in IL-10 reflects a reduction in the number of Tregs. IL-10 is a cytokine that modulates proinflammatory responses and when released by Tregs, may help to reduce secondary injury associated with inflammation during pathogen clearance (Maynard et al., 2007). Although our study was not designed to assess inflammatory pathways, a reduction in the ability of Tregs to release IL-10 suggests an impaired functional capacity that may extend to other suppressive functions of Tregs. Animal models that lack Tregs (i.e., scurfy mice) develop and succumb to autoimmune disease shortly after being weaned and autoimmune disease in humans is thought to arise, in part, by alterations to Tregs or to tolerance, which is a process by which unresponsiveness to self-antigens is developed. Reports of subsets of autistic patients that have increases in brain-specific autoantibodies (Silva et al., 2004; Wills et al., 2009) suggest that alterations to Tregs or the development of tolerance may contribute to an autistic phenotype. In addition, numerous studies have suggested that autism may arise from exposure to environmental agents that alter developmental processes. Our data demonstrate both PFOA and PFOS may affect the ability of ex vivo CD4+CD25+ Tregs to release IL-10, but that only PFOS may affect the number of Tregs. Additional work is required to further elucidate this relationship

Recommended changes to future work

Autism likely arises from causative events that increase the risk to triggering events. A cause without a trigger and a trigger without a cause would not lead to an autistic phenotype. Developmental exposure to environmental contaminants such as PFOA and PFOS may be a sufficient causative event, but because we did not apply a potential trigger, we were not able to induce changes to brain morphology. Several studies have hypothesized that post-natal events, such as exposure to pathogens or environmental agents, trigger underlying immune dysfunction induced by other agents during gestational development (summarized in Dietert and Dietert, 2008). In a repeat study of this experiment, we would expose the offspring to a trigger, such as lipopolysaccharide (LPS), during the lactation period. In this model, we would have both a potential causative agent and a post-natal trigger. In addition to evaluating the endpoints that we examined in the current study, we would include other endpoints such as behavior or neurochemistry as these measures may be more sensitive than brain morphology. We also would evaluate the level of activated microglia in the brains of the offspring as microglia may be the immune cell in the brain that responds to immune alterations in the periphery.

So what do our results mean?

The results of the PFOA studies suggest that the functional capacity of Tregs is affected by developmental exposure in male offspring. Our data have laid the groundwork for additional studies with Tregs and brain development. The results of our PFOS studies support what we observed with PFOA, but indicate that PFOA and PFOS may act via slightly different mechanisms to alter immune system development. Work by other researchers suggests that differences between the ability of PFOA and PFOS to induce developmental toxicity are mediated by affinity for specific cellular receptors. Additional work is required to determine if this is true for developmental immunotoxicity potentially induced by these compounds.

At this point, it is difficult to frame the meaning of our results mean within the body of research surrounding autism. It is generally held that autism arises from a combination of genetic and environmental factors and that multiple pathways may give rise to an autism diagnosis. Do PFOA and PFOS increase autism risk in individuals who may be exposed to these compounds during development? The results of our current model suggest that if exposure occurs during development at levels below 2 mg/kg, no definitive changes indicative of autism exist. However, are C57Bl/6 mice genetically susceptible? Are post-natal triggers required to induce additional changes? While our results are far from predictive of risk, they do indicate that certain cells of the immune system can be altered by developmental exposure to these compounds. In genetically susceptible individuals who experience the “right” combination of causes and triggers, developmental exposure to PFOA or PFOS may tip the balance from health to disease. A recent report by Grandjean et al. (2011) concluded that elevated exposure to perfluorinated compounds (including PFOA and PFOS) was associated with a reduced response to routine childhood vaccinations in children aged five and seven years. If the ability of the immune system to respond to pathogens is altered by exposure to these compounds, it is possible that other functions of the immune system, i.e., neurological development, can be altered as well.

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APPENDIX 1
BIBLIOGRAPHY OF MEETING ABSTRACTS

NeuroToxicology Conference, October 30-November 2, 2011.

Title: Immunopathogenesis in autism: regulatory T cells and the effects of developmental exposure to perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) on the cerebellum in C57Bl/6 mice

Authors: Ian Bryan and Jamie C. DeWitt

Abstract*:* Developmental exposure to exogenous agents may be associated with increased autism prevalence and may trigger underlying genetic susceptibilities toward the development of autism. The emerging contaminants perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) are widespread environmental pollutants that can induce developmental and immuno-toxicities. As immunopathologies have been reported in subsets of autistic patients, agents such as PFOA and PFOS can disrupt immune system development and may contribute to autism prevalence. An immunopathology associated with autism is the presence of serum autoantibodies against brain-specific proteins, which suggests damage to the brain during development via autoreactive T cells. Our hypothesis is that developmental exposure to PFOA or PFOS will lead to changes in levels of myelin basic protein (MBP) and may increase T cell infiltration in the cerebella. C57BL/6 female mice were orally exposed to 0.02, 0.2, or 2 mg/kg of PFOA, PFOS, or a water vehicle beginning at pairing with males and continuing through weaning of pups. In the mature male and female offspring, levels of MBP and T cell infiltration in the cerebella were evaluated. No dose-dependent changes in either endpoint were observed. Additional studies to evaluate the relationship between autoreactive T cells and neural development are in progress.

NeuroToxicology Conference, October 30-November 2, 2011.

Title: Immunopathogenesis in autism: regulatory T cells and markers of autoimmunity in mice developmentally exposed to perfluorooctanoic acid (PFOA)

Authors: Jamie C. DeWitt, Jason N. Franklin, and Qing Hu

Abstract*:* Developmental exposure to exogenous agents may be associated with increased autism prevalence and may trigger underlying genetic susceptibilities toward the development of autism. The emerging contaminant perfluorooctanoic acid (PFOA) is a widespread environmental pollutant that can induce developmental and immuno-toxicities. As immunopathologies have been reported in subsets of autistic patients, agents such as PFOA that can disrupt immune system development may contribute to autism prevalence. An immunopathology associated with autism is the presence of serum autoantibodies against brain-specific proteins, which suggests impacts to regulatory T cells (Tregs). Our hypothesis is that developmental exposure to PFOA will affect Tregs numbers and/or functions, increase autoimmune risk, and lead to changes in levels of myelin basic protein (MBP) in the brain. C57BL/6 female mice were orally exposed to 0.02, 0.2, or 2 mg/kg of PFOA or a water vehicle beginning at pairing with males and continuing through weaning of pups. In the immunocompetent male and female offspring, splenic Treg number and function, serum markers of autoreactivity, and levels of MBP and T cell infiltration in the cerebella were evaluated. In male offspring, all doses of PFOA decreased Treg ex vivo function by 70% on average, as measured by IL-10 release. Treg numbers were decreased by 6% in female offspring exposed to 2 mg/kg of PFOA. No effects were observed on serum markers of autoreactivity or in the cerebella. These data suggest that the functional capacity of Tregs may be undermined by developmental exposure to PFOA; however as no changes were observed in markers of autoreactivity in serum or brains, additional studies to evaluate vulnerable windows of exposure and the relationship between Tregs and neural development are in progress.

Society of Toxicology Conference, March 11-15, 2012.

Title: Regulatory T cells and markers of autoimmunity in mice developmentally exposed to perfluorooctane sulfonate (PFOS)

Authors: Jamie C. DeWitt, Jason N. Franklin, and Qing Hu

Abstract*:* Subsets of patients diagnosed with neurodevelopmental disorders have increases in serum autoantibodies against neural proteins, suggesting immunopathogenisis in the etiology of these disorders. As environmental pollutants such as perfluorooctane sulfonate (PFOS) can induce developmental and immuno-toxicities, developmental exposure to such agents may disrupt immune system development and contribute to altered neurodevelopment. The presence of serum autoantibodies against brain-specific proteins suggests impacts to regulatory T cells (Tregs). Our hypothesis is that developmental exposure to PFOS will affect Treg number and/or function and increase serum markers of autoreactivity. C57BL/6 female mice were orally exposed to 0.02, 0.2, or 2 mg/kg of PFOS or a water vehicle with 0.5% Tween beginning at pairing with males and continuing through weaning of pups. In immunocompetent male and female offspring, splenic Treg number and function and serum markers of autoreactivity were evaluated. Numbers of Tregs in male offspring at all doses of PFOS were suppressed by an average of 25% relative to control numbers ($P<0.05$). Treg numbers in female offspring were suppressed by 20% ($P<0.05$) only at the low dose. Decreases in treg function, as measured by IL-10 release from stimulated Tregs cultured with target cells ex vivo occurred only in females exposed to 0.02 mg/kg and in males exposed to 0.2 mg/kg. Serum markers of autoimmunity were not affected by dose. These data suggest that although numbers of Tregs may be susceptible by developmental exposure to PFOS, functional impacts may be preserved at the doses administered. Additional studies to evaluate vulnerable windows of exposure and the relationship between Tregs and neural development are in progress.